

Application No.: Not yet assigned
Preliminary Amendment dated February 23, 2004
Docket Number: 22311/04024

In the Specification:

Page 1, before "Background of the Invention" insert

Cross-Reference to Related Applications

This application is a continuation of the co-pending, commonly assigned, United States Patent Application Serial No.: 09/940,673, filed on August 27, 2001, which is a continuation of United States Patent Application Serial No.: 09/078,465, filed on May 14, 1998, which issued as United States Patent No.: 6,280,696, which is a continuation of United States Application Serial No. 08/203,532, filed on February 24, 1994, which issued as U.S. Patent No. 5,856,121 on January 5, 1999.

Please replace the paragraph at page 1, lines 12-24 and page 2, lines 1-6 with the following amended paragraph:

The deposits known as atherosclerotic plaques are comprised of lipoproteins, mainly cholesterol, proliferating vascular smooth muscle cells and fibrous tissue, and ~~extra-cellular~~ extracellular matrix components, which are secreted by vascular smooth muscle cells. As the plaques grow, they narrow the lumen of the vessel decreasing arterial blood flow and weakening the effected arteries. The resulting complications potentially include a complete blockage of the lumen of the artery, with ischemia and necrosis of the organ supplied by the artery, ulceration and thrombus formation with associated embolism, calcification, and aneurysmal dilation. When atherosclerosis causes occlusion of the coronary arteries, it leads to myocardial dysfunction, ischemia and infarction and often death. Indeed, 20-25% of deaths in the United States are attributable to atherosclerotic heart disease. Atherosclerosis also leads to lower extremity gangrene, strokes, mesenteric occlusion, ischemic encephalopathy, and renal failure, depending on the specific vasculature involved. Approximately 50% of all deaths in the United States can be attributed to atherosclerosis and its complications.

Please replace the paragraph at page 2, lines 23-29 with the following amended paragraph:

Surgical treatments include coronary artery bypass grafting, balloon angioplasty, or vessel endarterectomy which, when successful, bypass or unblock occluded arteries thereby restoring blood flow through the artery. The surgical treatments do not halt or reverse the

progression of the disease because they do not affect smooth muscle cell proliferation and secretion of ~~extra-cellular~~ extracellular matrix components.

Please replace the paragraph at page 3, lines 16-30 with the following amended paragraph:

Attempts have been made to prevent reocclusion of vessels after balloon angioplasties in experimental animals. One approach has been to treat rat carotid arteries with antisense oligonucleotides directed against the c-myb gene following balloon angioplasty de-endothelialization. In vascular smooth muscle cells the ~~The~~ expression of the c-myb gene is up-regulated during the G1 to S transition of the cell cycle, and the activation of c-myb expression is required for further cell cycle progression. The antisense oligonucleotides to c-myb blocked smooth muscle cell proliferation following balloon angioplasty. However, the antisense oligonucleotides are applied in a pleuronic gel to the adventitia, that is, the exterior, rather than the lumen side of the affected vessel. Exposing the the exterior of the vessel requires additional surgery with its attendant risks, and is therefore not desirable.

Please replace the paragraph at page 4, lines 15-25 with the following amended paragraph:

FIG. 1 is the nucleotide sequence SEQ ID NO: 1 of rat Gax gene with the predicted amino acid sequence SEQ ID NO: 2 listed below the nucleotide sequence. The homeobox is indicated by a box, and the CAX nucleotide repeat, where X is either cytosine or guanine, is underlined. A polyadenylation signal is in boldface and italics. Putative consensus sites are indicated as follows: for phosphorylation by protein kinase C, circles; for cyclic AMP (cAMP)-dependent protein kinase, squares; for casein kinase II, diamonds; and for histone H1 kinase, triangles. Residues which could potentially be a target for either cAMP-dependent protein kinase or protein kinase C are both circled and boxed.

Please replace the paragraph at page 4, lines 28-30 with the following amended paragraph:

FIG. 3 is the nucleotide sequence SEQ ID NO: 3 of human Gax gene with the predicted amino acid sequence SEQ ID NO: 4 listed below the nucleotide sequence;

Please replace the paragraph at page 7, lines 26-32 and page 8, lines 1-14 with the following amended paragraph:

The molecular control of cellular proliferation is not well understood. A class of genes, known as Homeobox genes, encode a class of transcription factors which are important in embryogenesis, tissue specific gene expression and cell differentiation. The homeobox genes share a highly conserved 183 ~~nucleotide~~ nucleotide sequence that is referred to as the "homeobox". The homeobox encodes a 61 amino acid helix-turn-helix motif that binds to adenine and thymine rich gene regulatory sequences with high affinity. Several vertebrate homeobox proteins have been shown to be transcription factors required for expression of lineage-specific genes. The tissue-specific transcription factors bind to DNA and repress or induce groups of subordinate genes. Many, but not all of these homeobox genes are located in one of four major clusters known as Hox clusters, designated Hox-1, Hox-2, Hox-3 and Hox-4. The Hox genes are expressed in the developing embryo, in distinct overlapping spatial patterns along the anterior-posterior axis which parallels the Hox gene order along the chromosome. Homeobox transcription factors control axial patterning, cell migration and differentiation in the developing embryo and are involved in the maintenance of tissue specific gene expression in adult organisms.

Please replace the paragraph at page 8, lines 23-28 with the following amended paragraph:

An adult rat aorta cDNA library in .lambda. ZAP, from Stratagene, was screened with a 64-fold degenerate 29-mer oligonucleotide containing three inosine residues directed at the most highly conserved region of the antennapedia homeodomain (helix 3), with the following sequence SEQ ID NO: 5, where I represents inosine:

Please replace the paragraph at page 8, line 36 through page 9, line 24 with the following amended paragraph:

The nucleotide sequence of the rat Gax gene SEQ ID NO: 1 is shown in FIG. 1. The cDNA encoding Gax is 2,244 base pairs in length, which corresponds to the size of the Gax transcript, that is the Gax mRNA, which is about 2.3 to 2.4 kb as determined by Northern blot analysis. The Gax cDNA has an open reading frame from nucleotide residues 197 to 1108

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beginning with an in-frame methionine that conforms to the eukaryotic consensus sequence for the start of translation and is preceded by multiple stop codons in all three reading frames. The open reading frame of the CDNA predicts a 33.6-kDa protein SEQ ID NO: 2 containing 303 amino acids with a homeodomain from amino acid residues 185 to 245, as shown in FIG. 1. To confirm that this cDNA was capable, of producing a protein product, the Gax open reading frame was fused in frame to the pQE-9 E. coli expression vector, from Qiagen, Inc., Chatsworth, Calif. and expressed in bacteria according to Hochuli, E., et. al. (1988) "Genetic Approach to Facilitate Purification of Recombinant Proteins with. a Novel Metal Chelate Adsorbent" Bio/Technology Vol. 6, pp. 1321-1325. E. coli containing this plasmid expressed a new ~~phosphorylated~~ protein of about 30 to about 36 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and extracts from these E. coli cells displayed a weak binding activity for the adenine and thymine rich, MHox-binding site in the creatine kinase M enhancer.

Please replace the paragraph at page 13, lines 12-19 with the following amended paragraph:

The nucleotide sequence SEQ ID NO: 3 of the human Gax gene coding sequence is shown in FIG. 3. Approximately 1.times.10.sup.6 plaques from a human genomic library in .lambda.FixII available from Stratagene were screened by conventional methods with a random primed EcoRI/BstXI fragment encompassing nucleotides 485-1151 of the rat Gax cDNA. Two clones contained the second exon of human Gax gene, having 182 base pairs. Using this coding information, the rest of the coding region was cloned by polymerase chain reaction methods.

Please delete line 31 on page 15.

Please replace the paragraph at page 16, lines 5-14 with the following amended paragraph:

Primer	Sequence 5'-3'
P2B	<u>SEQ ID NO:6</u> TCA, IA (G/A) , (G/A) TG, IGC, (G/A) TG, (T/C) TC
H2	<u>SEQ ID NO:7</u> GCGCGC (AGATCT) CAC, TGA, AAG, ACA, GGT, AAA
H2R	<u>SEQ ID NO:8</u> TT, TAC, CTG, TCT, TTC, AGT, GAG
H3	<u>SEQ ID NO:9</u> GCGCGC (AGATCT) AG, ATT, CAC, TGC, TAT, CTC, GTA
H6	<u>SEQ ID NO:10</u> GCGCGTGCCCCCTCTGATG, CTG, GCT, GGC, AAA, CAT, GT
H7	<u>SEQ ID NO:11</u> GCGCGC (TCTTGA) AGG, GCG, AGA, GAG, GAT, TGG, GA
AP	<u>SEQ ID NO:12</u> CTGGTTTCGCCCCACCTCTGAAGGTTCCAGAATCGATAG
Anchor	<u>SEQ ID NO:13</u> GGAGACTTCCAAGGTCTTAGCTATCA (CTTAAG) CAC

Engineered enzyme sites are bracketed.

Please replace the paragraph at page 18, lines 25-31 and page 19, lines 1-22 with the following amended paragraph:

The cultured cells were exposed to various mitogens as discussed below. The cells were then harvested and the total mRNA was extracted. The total RNA from rat cultured cells was prepared by the guanidine thiocyanate method according to Chomczynski, P., and N. Sacchi, (1987) "Single-step Method of RNA Isolation by Acid Guanidinium Thiocyanate-phenol-chloroform Extraction" Anal. Biochem. Vol. 162, pp. 156-159, fractionated on 1.2% agarose gels containing formaldehyde, and blotted onto nylon membranes. The RNA from cultured cells was separated on 30-cm gels for transcript size determination and on 10-cm gels for other studies. Hybridizations were carried out at 65.degree. C. in buffer containing 0.5 M sodium phosphate at pH 7.0, 7% sodium dodecyl sulfate, 1 mM EDTA, and 1% bovine serum albumin, using a cDNA probe labeled by random priming consisting of a truncated Gax cDNA lacking the 5' end and the CAX repeat, where the X may be cytosine or guanine. Probes for Hox-1.3 and Hox-1.4 consisted of the cDNAs isolated from the rat aorta library, and the probe for Hox-1.11 consisted of the DraI-EcoRI fragment of its cDNA. The blots were washed with a final stringency of 0.1 to 0.2.times.SSC-0.1% sodium dodecylsulfate at 65.degree. C. After the probings with the homeobox probes were complete, the blots were rehybridized with a probe to rat glyceraldehyde 3-phosphate dehydrogenase hereinafter also referred to as "GAPDH," to demonstrate message integrity. Gax mRNA and GAPDH mRNA were quantified with a Molecular Dynamics model 400S PhosphorImager to integrate ~~bank~~ band intensities, or by scanning densitometry of autoradiograms. In all quantitative comparisons of Gax mRNA levels between experimental groups, Gax mRNA levels were normalized to the corresponding GAPDH level determined on the same blot, to account for differences in RNA loading.

Please replace the paragraph at page 24, lines 1-9 with the following amended paragraph:

To produce the Gax protein evaluated herein, the cDNA coding regions for Gax was fused in frame to the pGEX-2T expression vector obtained from Pharmacia Biotechnology, and then expressed in E. coli. Specifically, GST-Gax was produced according to the following procedure: the coding region of Gax cDNA spanning from nucleotides 200-1108 was amplified by polymerase chain reaction methods using the following primers:

5'GCGCGCGTCGACGAACACCCCCTCTTTGGC 3' SEQ ID NO: 14 and
5'GCGCGCAAGCTTTCATAAGTGTGCGTGCTC 3' SEQ ID NO: 15

Please replace the paragraph at page 25, lines 16-21 with the following amended paragraph:

Microinjections were performed using a semiautomatic microinjection system from Eppendorf Inc. in conjunction with a Nikon Diaphot phase contrast microscope. According to Peperkole, R., et al. (1988) Proc. Natl. Acad. Sci. USA Vol. 85, pp. 6758-~~6752~~6762, The injection pressure was set at 70-200 hPa and the injection time was 0.3 to 0.6 seconds.

Please replace the paragraph at page 31, lines 1-14 with the following amended paragraph:

SV40-transformed vascular smooth muscle cell proliferation was inhibited by GST-Gax protein, as shown in Table 3. The GST-Gax protein also inhibited the proliferation of fibroblast cell lines NIH3T3 and BALB/c 3T3. GST-Gax protein also inhibited the proliferation of human cells, specifically human vascular smooth muscle cells and human foreskin fibroblasts. These results indicate that Gax action is not cell type-specific, although there are differences in the extent inhibition among the different cell types. ~~The~~ Among the human cells, the GST-Gax protein exhibits maximal inhibition in vascular smooth muscle cells, the cell type in which the Gax gene is normally expressed. Similarly among the rat cells, the GST-Gax protein exhibits maximal inhibition in vascular smooth muscle cells, the cell type in which the Gax gene is normally expressed.

Please replace the paragraph at page 33, lines 1-33 and page 34, lines 1-8 with the following amended paragraph:

The Gax gene expression in normal blood vessels and in injured blood vessels was compared to determine whether Gax gene down-regulation occurs in response to injury-induced smooth muscle cell proliferation in vivo. Adult male Sprague-Dawley rats were subject to acute vessel injury by balloon de-endothelialization in the carotid arteries according to the methods of Majesky, M. W., et al. J. Cell. Biol. (1990) Vol. 111, pp. 2149-2158. The expression levels of Gax, that is, the mRNA levels, were assessed relative to that of glyceraldehyde 3-phosphate dehydrogenase (hereinafter also referred to as "G3") by a quantitative polymerase chain reaction.

At various times following balloon de-endothelialization the rats were sacrificed and the total RNA was isolated from the vascular smooth muscle tissues using the TRI reagent from Molecular Research Center, Inc. The cDNA was synthesized from the extracted RNA with MMLV reverse transcriptase from Bethesda Research Labs. Aliquots of the cDNA pools were subjected to polymerase chain reaction amplification with AmpliTaq DNA polymerase from Perkin-Elmer in the presence of α -³²P-dCTP with the following cycle conditions: 94.degree. C. for 20 seconds, 55.degree. C. for 20 seconds, and 72.degree. C. for 20 seconds. The final cycle had an elongation step at 72.degree. C. for 5 minutes. The primers for the rat Gax amplification were: 5'-CCCGCGCGGCTTTTACATTAGGAGT-3' SEQ ID NO: 16 and 5'-GCTGGCAAACATGCCCTCCTCATTG-3' SEQ ID NO: 17. The primers for the rat G3 gene were 5'-TGATGGCATGGACTGTGGTCATGA-3' SEQ ID NO: 18 and 5'-TGATGGCATGGACTGTGGTCATGA-3' SEQ ID NO: 19. The Gax cDNA was amplified for 30 cycles, and G3 was amplified for 25 cycles in the same reaction vessels. The amount of a radioactive label incorporated into the amplified cDNA and G3 fragments was determined by subjecting the fragments to electrophoresis on a 1% agarose gel, then excising the bands and liquid scintillation counting. Since the mRNA levels of glyceraldehyde 3-phosphate dehydrogenase remain relatively constant following this procedure (see J. M. Miano et al. 1990, Am. J. Path. 137, 761-765), the ratio of radiolabel incorporation into the Gax-derived amplified bands and the G3-derived amplified bands corrects for differences arising from the efficiency of RNA extraction from the different animals, and it provides a measure of Gax mRNA levels in the normal and injured vascular tissues. These ratios are plotted in FIG. 14.